Veterinär-Anatomisches Institut

der Vetsuisse-Fakultät, Universität Zürich

Direktor: Prof. Dr. med. vet. Alois Boos

Arbeit unter wissenschaftlicher Betreuung von PD Mariusz P. Kowalewski, PhD

Biosynthesis and degradation of canine placental prostaglandins: Expression and function of prostaglandin F2alpha-synthase (PGFS) and 15-prostaglandin dehydrogenase (15PGDH)

Inaugural Dissertation

zur Erlangung der Doktorwürde der
Vetsuisse-Fakultät, Universität Zürich

vorgelegt von

Aykut Gram

Tierarzt
aus Samsun, Türkei

genehmigt auf Antrag von

PD Mariusz P. Kowalewski, PhD, Referent

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1 Summary

There exists no distinct explanation for the prepartal prostaglandin F2α (PGF2α) increase mechanism in dogs. While the PGF2α-synthase (PGFS) mRNA expression and localization profiles have been previously investigated in canine utero/placental compartment, the availability and biochemical activity of the PGFS protein remains unknown. Consequently, in order to provide a basis for better understanding of mechanisms controlling the provision of prostaglandins, canine specific PGFS- and 15-Prostaglandin Dehydrogenase (15PGDH)-antibodies were generated and used in the present study. The spatio-temporal expression and biochemical activities of recombinant canine PGFS and 15PGDH were investigated throughout pregnancy.

From the strong expression of PGFS observed in the utero/placental compartment during post implantation and mid-gestation, which reflects the mRNA-levels observed previously, and its localization in the placenta fetalis, the possible role of PGF2α in the trophoblast invasion during canine placentation is suggested. Similar localization pattern, together with the increased expression from pre-implantation until mid-gestation, were observed for 15PGDH.

Cumulatively, the presented results imply a possible functional interplay between PGFS and 15PGDH controlling the placental development and prepartum PGF2α output in the dog. 15PGDH appears to be a potential "gate-keeper" in the local provision of prostaglandins from the pregnant canine uterus.

2 Zusammenfassung

Titel:

Synthese und Abbau plazentärer Prostaglandine bei der Hündin: Expression und Funktion der Prostaglandin-F2α-Synthase (PGFS) und der 15-Prostaglandindehydrogenase (15PGDH).

Die Ursache des präpartalen Prostaglandin F2α (PGF2α)-Anstiegs bei der Hündin ist noch weitgehend unklar. Während die PGFS-mRNA-Expressions- und -Lokalisations-Muster im kaninen utero/plazentären Kompartiment jüngst erforscht wurden, sind die Verfügbarkeit und Aktivität der PGFS weiterhin nicht bekannt. Daher wurden hundespezifische PGFS- und 15PGDH-Antikörper hergestellt und in der vorliegenden Studie verwendet, um die Mechanismen, welche die Verfügbarkeit von Prostaglandinen regulieren, besser kennen zu lernen.

Das zeitliche und räumliche Muster der Expression und Aktivität der rekombinanten PGFS und 15PGDH wurden im Trächtigkeitsverlauf untersucht. Die starke Expression der PGFS, welche im utero/palzentären Gewebe während der Postimplantation und der Trächtigkeitsmitte beobachtet wurde, die auch die jüngst ermittelten mRNA-Mengen widerspiegelt, und ihre Lokalisation in der Plazenta fetalis deuten insgesamt auf eine mögliche Rolle des PGF2α bei der Trophoblasteninvasion während der Plazentation beim Hund hin. Ein ähnliches Verteilungsmuster, zusammen mit der ansteigenden Expression von der Präimplantation bis zur Mitte der Trächtigkeit, wurde auch bei der 15PGDH beobachtet.

Die vorliegenden Resultate deuten auf ein mögliches Zusammenspiel zwischen der PGFS und der 15PGDH bei der Steuerung der Plazentation und dem präpartalen PGF2α-Anstieg beim Hund. Die 15PGDH scheint ein möglicher Regulator bei der lokalen Bereitstellung von Prostaglandinen im Uterus der tragenden Hündin zu sein.

3 Authors and correspondence

Biosynthesis and degradation of canine placental prostaglandins: Expression and function of prostaglandin F2alpha-synthase (PGFS) and 15-prostaglandin dehydrogenase (15PGDH).

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Short title: Expression and function of canine placental PGFS and 15PGDH

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4 Abstract

There is no distinct explanation of the mechanism for the prepartal prostaglandin F2alpha (PGF2alpha) increase in pregnant dogs. While the *PGF2alpha-synthase* (*PGFS*) mRNA expression and localization profiles have been previously investigated in canine utero/placental compartments, the availability and biochemical activity of the PGFS protein remain unknown. Consequently, in order to provide a basis for better understanding of mechanisms controlling the provision of prostaglandins, canine specific PGFS and 15-Prostaglandin Dehydrogenase (15PGDH) antibodies were generated and used in the present study. The expression, cellular localization and biochemical properties of PGFS and 15PGDH were investigated in the utero/placental compartments and corpus luteum (CL) throughout pregnancy and at the prepartum luteolysis. The utero/placental PGFS-expression was upregulated during post-implantation and mid-gestation followed by a prepartal decrease; only weak or no signals were observed in samples derived from luteal tissues. The utero/placental expression was localized in the superficial uterine glands throughout gestation and in the trophoblast cells within the feto-maternal contact zone during placentation, indicating its possible role in the trophoblast invasion during canine placentation. 15PGDH was upregulated until mid-gestation; the prepartal drop observed afterwards was significant. Cellular localization was in the endometrial surface- and glandular-epithelia. Positive signals were observed in the trophoblast cells at the feto-maternal contact zone. The biochemical activity of recombinant PGFS and 15PGDH was confirmed after its expression in a heterologous system. The colocalization of 15PGDH with the PGFS expression suggests its modulatory role as a possible “gate keeper” of the supply of prostaglandins from the pregnant canine uterus.

5 Introduction

Domestic dogs are monoestrous, predominantly non-seasonal breeders with an obligatory anoestrus period between active reproductive phases. The progesterone (P4) of luteal origin is required for maintenance of pregnancy, since the corpora lutea (CL) are the only source of this hormone during pregnancy and in non-pregnant cycles. CL reveal an almost identical endocrine profile in both situations that reaches maximal P4 levels in peripheral blood within the first 20-25 days of dioestrus or pregnancy (reviewed in (Hoffmann et al., 2004)). Afterwards, P4 starts to decline progressively until concentrations <1ng/ml are reached in non-pregnant dogs, signalling the onset of anoestrus (Concannon, 1993). There are no indications of an active luteolytic principle in non-pregnant bitches that would involve the uterine PGF2alpha; on the contrary, normal ovarian function is maintained following hysterectomy (Hoffmann et al., 1992). Similarly, the role of the locally produced prostaglandins seems to be directed towards the para/autocrine regulation of CL formation rather than towards luteolysis (Kowalewski et al., 2006b; Kowalewski et al., 2008). Consequently, the slow luteal regression observed in non-pregnant bitches appears to be a passive degenerative process that is likely attributable to aging of the CL (reviewed in (Hoffmann et al., 2004)). Other than that, in pregnant animals the initial slow decrease of P4 suddenly becomes a dramatic drop approximately 24-48h before parturition, during the acute prepartum luteolysis. This is accompanied by a strong increase of the PGF2alpha-metabolite (13,14-dihydro-15-keto-PGF2alpha; PGFM) in maternal peripheral blood (Nohr et al., 1993; Hoffmann et al., 1994), which in turn, strongly implies a possible direct role of PGF2alpha during the prepartum luteolysis and/or in fetal expulsion. The prepartal PGF2alpha release may originate in the fetal part of the placenta, more specifically in the fetal trophoblast cells where strongly upregulated expression of PTGS2 (formerly known as COX2) was observed (Kowalewski et al., 2010). Similar observation has been made in experiments with an

antigestagen, where blocking of the P4-receptor resulted in a significant upregulation of PTGS2 in the utero/placental compartment, predominantly in the fetal trophoblast (Kowalewski et al., 2010). Assuming that PTGS2 is the rate-limiting factor, the placental prostaglandin system especially appears to be strongly activated at the time of the prepartum PGF2alpha output. This hypothesis corroborates the findings by Luz et al. (Luz et al., 2006) who postulated the placenta as the main source of the luteolytic PGF2alpha in the dog. Consequently, based on the data available to date, in contrast to non-pregnant dogs, prepartal luteolysis appears to be an active process of CL destruction by PGF2alpha originating in the pregnant uterus.

Recently, the canine *PGFS* cDNA sequence has been cloned (Kowalewski et al., 2008). Making use of its availability, the respective mRNA-expression profiles were examined in canine reproductive tissues throughout pregnancy (Kowalewski et al., 2010). Interestingly, the highest expression of *PGFS* mRNA in the utero/placental compartments was observed during post-implantation and mid-gestation; intriguing was the prepartum decline that was observed at the concomitant PGF2alpha increase in peripheral blood. This prepartal increase in PGF2alpha led us to speculate on possible mechanisms regulating its generation. Thus, the post-transcriptional regulation and enhanced substrate turnover, due to the elevated availability of PTGS2, could be important factors in elevating the PGF2alpha levels during prepartum luteolysis. (Kowalewski et al., 2010). However, due to the lack of canine specific and/or cross-reacting antibodies, corresponding data on PGFS expression at the protein level could not be collected in this earlier study. Consequently, no final conclusions could be drawn. It is noteworthy that the cloned canine PGFS is a member of the aldo-keto reductase family which includes a number of closely-related enzymes sharing a very high (over 80%) mRNA and protein homology. Therefore, until the biochemical properties and substrate specificity of the respective PGFS protein towards the conversion of PGH2 to PGF2alpha

have been elucidated, the aforementioned data on the mRNA expression profiles need to be treated cautiously.

The biological availability of prostaglandins can be regulated by the activity of 15-hydroxy prostaglandin dehydrogenase (15PGDH), an enzyme that catabolizes PGE2 and PGF2alpha to their inactive metabolites, PGFM and 13,14-dihydro-15-keto PGE2 (PGEM) (Okita and Okita, 1996; Kankofer, 1999). *15PGDH* was cloned from a number of mammalian tissues, e.g. cattle, mouse, human, sheep, horse and rat (Ensor et al., 1990; Matsuo et al., 1996; Zhang et al., 1997; Silva et al., 2000; Parent et al., 2006; Sayasith et al., 2007). There are, however, no available data on the expression and regulation of *15PGDH* in the reproductive tissues of the dog. Especially in terms of the post-transcriptional regulation of PGF2alpha activity, 15PGDH could be an important para/autocrine factor controlling the low PGFM levels detected in peripheral plasma at the time of increased placental *PGFS* mRNA expression. In turn, the decreased functional bioavailability of 15PGDH prior to parturition could facilitate increased output of PGF2alpha into the peripheral blood.

Taking all of the above under consideration and in order to better understand the regulatory mechanisms governing the provision of PGF2alpha in the bitch, the objectives of the present study were to generate canine-specific anti-PGFS and 15PGDH antibodies, to validate them and, finally, to apply them for investigations into the spatio-temporal expression of the respective proteins within the canine CL and utero/placental compartments throughout pregnancy and at the prepartum luteolysis. The biochemical properties of the heterogeneously expressed recombinant canine PGFS and 15PGDH proteins were tested on their specific substrates.

6 Material and Methods

6.1 Animals, Tissue Sampling and Preservation

Eighteen crossbreed, healthy bitches, aged 2-8 years, were used for this study. All experiments were conducted in accordance with animal welfare legislation. Four groups were created with dogs ovariohysterectomized according to the following schedule:

Group 1: pre-implantation, days 8-12, n=5

Group 2: post-implantation, days 18-25, n=5

Group 3: mid-gestation, days 35-45, n=5

Group 4: prepartal luteolysis, n=3

The day of mating was recorded as Day 0 for all bitches. In the pre-implantation group, pregnancies were confirmed by uterine flushings. The time of prepartal luteolysis was detected by measuring P4 levels at 6 h intervals from Day 58 of pregnancy onwards. When a steep P4 decline (below 3ng/ml) was detected in three consecutive measurements, females were subjected to ovariohysterectomy.

For immunohistochemistry (IHC) and in situ hybridization (ISH), immediately after surgery, the CL and utero/placental compartments were trimmed of surrounding connective tissues and fixed in 10% neutral phosphate buffered formalin for 24 h at +4^{o}C. Afterwards, tissue samples were washed with phosphate buffered saline (PBS) that was frequently changed during one week, dehydrated in a graded ethanol series and embedded in paraffin-equivalent Histo-Comp (Vogel, Giessen, Germany).

For RNA preservation and western blot analyses, samples were immediately soaked in RNAlater (Ambion Biotechnology GmbH, Wiesbaden, Germany) and incubated for 24 h at +4^{o}C. For long-term storage tissue samples were held at -80°C.

As already described (Kowalewski et al., 2010), the following average P4 concentrations were measured at the respective periods of pregnancy: 35.71 ± 7.9 ng/ml at the pre-implantation period, 29.73 ± 13.23 ng/ml at post-implantation, 13.32 ± 8.66 ng/ml at midgestation and 2.07 ± 0.99 ng/ml during the prepartum luteolysis.

6.2 RNA isolation, reverse transcription and homology cloning of canine specific 15PGDH

Total RNA was isolated from utero/placental compartments and CL using TRIZOL®-Reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). The RNA content was measured with a SmartSpec™ Plus spectrophotometer (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Prior to reverse transcription (RT), in order to remove genomic DNA contamination, DNAse treatment with RQ1 RNase-free DNase (Promega, Dübendorf, CH) was performed following the manufacturer's protocols. Subsequently, applying our previously published protocol (Kowalewski et al., 2006b) and using random hexamers as primers, complementary DNA (cDNA) was synthesized with RT reagents purchased from Applied Biosystems (Foster City, CA, USA). Between 100-200 ng of DNase-treated total RNA were used for each sample in RT reactions carried out in an Eppendorf Mastercycler ® (Vaudaux-Eppendorf AG, Basel, CH) was used. The RT PCR conditions were as follows: 8 min at 21°C, 15 min at 42°C, then the reaction was stopped by incubation for 5 min at 99°C.

The canine specific *15PGDH* cDNA sequence had not been characterized prior to our study. Hence, molecular cloning and sequencing became prerequisites in order to obtain the anticipated data on the mRNA level. Based on the alignment of the known *15PGDH* homologues with an online available canine genomic sequence, the following primers flanking the predicted open reading frame (ORF) of canine *15PGDH* were chosen: forward: 5'-ATG CAC GTG AAC GGC AAA GT- 3' and reverse: 5'-CTG AGT TTT TGC ATG

AAA TG-3' and ordered from Microsynth AG (Balgach, CH). The GeneAmp Gold RNA PCR Kit from Applied Biosystems was applied as described previously (Kowalewski et al., 2006b) for the hot-start PCR reaction. The annealing temperature was 58°C. Using the RNA isolated from canine CL and utero/placental compartments of at least three dogs, PCR products comprising 801 bp of canine *15PGDH* ORF coding for 267 amino acids were successfully amplified. The subsequent cloning procedure consisted of separating PCR products on a 2% ethidium bromide stained agarose gel, extracting them using the Qiaex II gel extraction system (Qiagen GmbH Hilden, Germany), subcloning into the pGEM-T vector (Promega), transforming and amplifying in XL1 Blue competent cells (Stratagene, La Jolla, CA, USA), purifying bacterial plasmids with the PureYield™ Plasmid Midiprep System (Promega) and finally sequencing on both strands with T7 and Sp6 primers (Microsynth). Subsequently, the cloned sequence was submitted to GenBank with accession number: JQ407019: Canis lupus familiaris 15-hydroxyprostaglandin dehydrogenase mRNA, complete cds. ChromasPro Version 1.2 oligo software (Technelysium Pty. Ltd.) was used for prediction of the amino acid sequence.

6.3 Real Time (TaqMan) PCR

Semi-quantification of *15PGDH* mRNA expression in the utero/placental compartments and CL throughout pregnancy was performed by means of real-time (TaqMan) PCR analysis using an automated fluorometer ABI PRISM 7500 Sequence Detection System (Applied Biosystems). Each sample was quantified in duplicate using 96-well optical plates (Applied Biosystems).

The DNase treatment and cDNA synthesis were as for qualitative PCR and as described above. For negative controls, autoclaved water instead of cDNA and the so-called 'minus RT control' were used. The reaction mixtures of 25µl were prepared as follows: 200nM TaqMan

Probe, 300 nM of each primer, 12.5 µl Fast Start Universal Probe Master (ROX)® (Roche Diagnostics, Mannheim, Germany) and 5µl cDNA corresponding to 100nm total RNA per sample in duplicate. The amplification conditions were: denaturation at 95 °C for 10 min, 40 cycles at 95°C for 15 sec and 60°C for 60 sec. The semi-quantitation of *15PGDH* mRNA expression was obtained by using three reference genes, *GAPDH*, *18SrRNA* and cyclophilin, in the comparative CT method (ΔΔCT method) according to the manufacturer's protocols for the ABI 7500 Fast Real-Time PCR System and as described previously (Kowalewski et al., 2006b; Kowalewski et al., 2011). The CT slope method was applied for measurement of the efficiency of the PCR assays that were established to ensure approximately 100% reaction efficiency. Primers and 6-carboxyfluorescein (6-FAM)- and 6-carboxytetramethyl-rhodamine (TAMRA)-labelled TaqMan probes were designed using Primer Express Software (Version 2.0, Applied Biosystems) and purchased from Microsynth (Balgach, CH). The sequences were as follows: *15PGDH* forward: 5'-GGC AGC GAA TCT CAT GAA CAG-3', *15PGDH* reverse: 5'- TCT TCT TTC TCA ATG GAT TCA AGGA-3', *15PGDH* TaqMan probe: 5'-TGA ATG CCA TTT GCC CAG GCT TTG T-3'; length of the amplicons was 93bp. *GAPDH* forward: 5'-GCT GCC AAA TAT GAC GAC ATC A-3', *GAPDH* reverse: 5'-GTA GCC CAG GAT GCC TTT GAG-3', *GAPDH* TaqMan probe: 5'-TCC CTC CGA TGC CTG CTT CAC TAC CTT-3' (GenBank accession number AB028142); length of the amplicons 75bp. *18SrRNA* forward: 5'-GTC GCT CGC TCC TCT CCT ACT-3', *18SrRNA* reverse: 5'-GGC TGA CCG GGT TGG TTT-3', *18SrRNA* TaqMan probe: 5'-ACA TGC CGA CGG GCG CTG AC-3' (GenBank accession number FJ797658); length of the amplicons 125bp. A commercially available canine-specific *cyclophilin A* TaqMan system was purchased from Applied Biosystems (Foster City, CA, USA; Prod. No. Cf03986523- gH).

6.4 Construction of Expression Plasmids

The PGFS- and 15PGDH- expression plasmids were cloned by means of Gateway cloning Technology (Invitrogen) following the manufacturer's protocol. The templates used were canine-specific sequences of *PGFS* (GenBank accession number: AY875970: Canis lupus familiaris prostaglandin F synthase (*PGFS*) mRNA, complete cds; (Kowalewski et al., 2008) and the freshly cloned *15PGDH* sequence (GenBank: JQ407019). In the first step, attB sequences were added to *PGFS* and *15PGDH* forward and reverse primers for generating *PGFS* and *15PGDH* ORF encoding PCR products flanked by attB recognition sites for BP recombination. Stop codons were omitted in the reverse primers to generate templates for the subsequent cloning procedure of fusion proteins expressing constructs; regions of gene-specific sequence are underlined:

attB1-*PGFS* forward: 5'-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT AAT GAA TCT AAT GAA ACT TAG-3'

attB2-*PGFS* reverse : 5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTT ATC CTC GTC ATT AAA TG-3'

attB1-*PGDH* forward: 5'-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT AAT GCA CGT GAA CGG CAA AGT-3'

attB2-*PGDH* reverse: 5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTT CTG AGT TTT TGC ATG AAA TG- 3'

The reaction mixture was prepared as follows: 12.5 µl water, 10µl 5x Phusion HF Buffer, 200µM dNTP-mix, 0.5 µM forward primer sense, 0.5µM reverse primer, 5% DMSO, 0.75 M betaine (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland), 1U Phusion® High-Fidelity DNA hot start Polymerase and 25mM $MgCl_2$ (New England Biolabs, Frankfurt, Germany). Amplification conditions were: initial denaturation at 98°C for 1min, followed by 34 cycles at 98°C for 10 sec, 50°C for 20 sec, 72°C for 30 sec, and final elongation at 72°C for 5min. Reactions were carried out in an Eppendorf Mastercycler ® (Vaudaux-Eppendorf AG). After

separation at 100V on an ethidium bromide-stained 1% agarose gel, PCR products were purified using the Qiaex II gel extraction system (Qiagen GmbH) according to the manufacturer's instructions. Afterwards, following the Gateway system manufacturer's protocol, BP recombination was performed using the BP clonase II between an attB-flanked cDNA fragment and attP-containing pDONR221 vector to produce an entry clone. The entry clone was transformed in One Shot® ccdB Survival™ 2 T1R Competent Cells (Invitrogen). Subsequently, bacteria were plated onto LB-Agar-plates containing 50μg/ml Kanamycin (Sigma-Aldrich). This was followed by isolation of entry clones using a PureYield™ Plasmid Miniprep system (Promega) and control restriction digestion. In the next step, LR recombination was performed with pHSV-EYFP-Rfc-C1 and pHSV-V5His-Rfc-C1 expression vectors (Laimbacher, 2006) in order to generate plasmids expressing the EYFP (Enhanced Yellow Fluorescent Protein) and His-tagged canine specific PGFS and 15PGDH fusion proteins. The LR recombination was performed between the entry clone's attL sites and attR sites existing in the expressing vectors, using LR clonase II (Invitrogen). Afterwards, plasmids were transformed into One Shot® ccdB Survival™ 2 T1R Competent Cells (Invitrogen) and plated on LB-Agar-plates containing 100ug/ml Ampicillin (Sigma-Aldrich). Purification of plasmids with PureYield™ Plasmid Miniprep (Promega), control restriction digestion and subsequent sequencing on both strands (Microsynth) of plasmids expressing recombinant canine PGFS- and 15PGDH-, His- and EYFP-tagged fusion proteins completed the cloning procedure. Finally, the plasmids were amplified and isolated using the Plasmid Maxi Kit from Qiagen according to the manufacturer's instructions.

6.5 Cell cultures and transient expression

The Vero 2.2-cell line was used as a heterologous system and was cultured in Dulbecco's Modified Eagle's Medium (DMEM)-high glucose (PAA, Pasching, Austria) with 2mM L-

glutamine including 2% fetal bovine serum (PAA) and 1% penicillin-streptomycin (PAA). Prior to transfection, cells were seeded into 100 mm Petri dishes. After the Vero 2.2-cells reached 80% confluency, transfection was performed with pHSV-EYFP-Rfc-C1-PGFS or -15PGDH and pHSV-V5His-Rfc-C1-PGFS or -15PGDH expression vectors to produce recombinant canine EYFP- and His-tagged PGFS and 15PGDH fusion proteins. The FuGENE® HD-transfection reagent (Roche Diagnostics) was used according to the manufacturer's protocol in 3ml serum-containing medium at a ratio of 1μg DNA to 3.5μL reagent, in complexes that were incubated at room temperature for at least 25 min in 100μL serum-free medium. Subsequently, cells were incubated at 37°C in humidified air containing 5% CO2. Six hours after transfection, the medium was changed and cell culture was continued for another 24 hours. Transfection efficiency was assessed by monitoring the cellular expression of EYFP-tagged PGFS- and 15PGDH- proteins under a fluorescent microscope. For harvesting, cells were rinsed with ice-cold PBS and lysed using 300μl of NET-2 lysis buffer (50mM Tris-HCl, pH 7.4, 300mM NaCl, 0.05% NP-40) containing 10μl/ml protease inhibitor cocktail (Sigma Aldrich).

6.6 Purification and assessment of biochemical activities of PGFS- and 15PGDH-His coupled recombinant proteins

Canine recombinant His-tagged PGFS and 15PGDH proteins were expressed in Vero 2.2 cells and purified with Ni-NTA (nickel-nitrilotriacetic acid) Magnetic Agarose Beads (Qiagen) following the manufacturer's protocol. Protein concentration was measured by a Bradford assay using the SmartSpec™ Plus spectrophotometer (Bio-Rad Laboratories). Activity of recombinant His-tagged PGFS protein was initiated by adding 100μM NADPH (Sigma Aldrich) to 100μl of mixtures containing 1μg recombinant purified canine PGFS-His, 50mM Tris HCl pH 7.5 and 10-30μM PGH2. For assessment of 15PGDH-His activity, reactions

were initiated by adding 1μM NAD^+ (Sigma Aldrich); 10-30μM PGE2 or PGF2alpha were used as substrates. All experiments were repeated at least three times. For negative controls, no recombinant proteins were used and the resulting background signals were subtracted from the values obtained for samples in which recombinant proteins were used. Reactions were run at 37°C for 1-10 min and finally heat-inactivated for 5 min. The conversions of PGH2 into PGF2alpha and PGE2, of PGF2alpha into PGFM and of PGE2 into PGEM, were assessed by using the PGF2alpha, PGE2, PGEM and PGFM EIA Kits from Cayman (Michigan, USA) following the manufacturer's instructions. The intra- and inter-assay CVs were approx 3%. In a final step, plates were read photometrically using the SpectraMax Plus384 Absorbance Microplate Reader (Bucher Biotec A. G., Basel, Switzerland) set at 405 nm.

6.7 Protein preparation and western blot

Transfected cells and tissue homogenates were prepared with NET-2 lysis buffer (50mM Tris-HCl, pH 7.4, 300mM NaCl, 0.05% NP-40) and protease inhibitor cocktail (10μl/ml) mixed solution. Subsequently, tissue extracts were centrifuged at 10,000 x g for 10 min at 4°C. Proteins in the supernatant were disrupted by sonication (Vibra-Cell, Newton, CT, USA) at 75W for 15s and solubilized in sample buffer (25mmol/L Tris-Cl, pH 6.8, 1% SDS, 5% ß-mercaptoethanol, 10% glycerol, 0.01% bromphenol blue). Protein concentration was measured by the Bradford assay with a SmartSpec™ Plus spectrophotometer (Bio-Rad). SDS-PAGE was performed on a 12% polyacrylamide gel (Bio-Rad) at 120V. Afterwards, proteins were transferred onto methanol-activated polyvinylidene difluoride (PVDF) (Bio-Rad) membranes for 1h at 100 V. Subsequently, the membranes were incubated for 1h in 5% low fat milk powder in PBST (0.25% Tween-20, PBS), to block non-specific binding. Membranes were then incubated overnight at 4°C in 2.5 % low fat milk powder in PBST containing a primary antibody. Afterwards, membranes were washed 3 x for 10 min in PBST at room

temperature on a shaker and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 1h at room temperature. After subsequent washing 5 x for 10 min in PBST, signals were initiated by adding the Immun-Star™ WesternC™ Chemiluminescent Kit substrate (Bio-Rad) according to the manufacturer's protocol. The blots were visualized with the ChemiDoc™ XRS+ System and Image Lab. Software, both from Bio-Rad.

Due to the lack of canine cross-reacting antibodies, canine-specific guinea pig polyclonal affinity purified anti-PGFS and -15PGDH, custom-made antibodies were generated (Eurogentec S.A, Seraing, Belgium) using the following peptide sequences: DTLFATHPDYPFNDED - C-terminal amino acids 309-324 of the canine PGFS sequence with GenBank accession Number: NP_001012344 and HFQDYETTPFHAKTQ - C-terminal amino acids 252-266 of canine 15PGDH sequence with GenBank accession number AFF60303. The antibodies were used at 1:1000 and 1:50 dilutions for PGFS and 15PGDH, respectively. The synthetic peptides used for immunizations were applied as epitope-specific blocking peptides. Additional antibodies used were: mouse monoclonal anti-EYFP (JL-8) from Clontech Laboratories (Saint-Germain-en-Laye, France; 632380; dilution 1:1000) and monoclonal mouse anti-His from Invitrogen (R940-25; dilution 1:100); ß-actin mouse monoclonal antibody (sc-69879; Santa Cruz Biotechnology, Rockford, IL, USA; dilution 1:1000); secondary HRP goat anti-mouse IgG (W402B) from Promega Corp. (Madison, WI, USA; dilution 1:15,000) and anti-guinea pig IgG conjugated to HRP from Sigma (A5545; dilution 1:15,000).

6.8 Immunohistochemistry

Formalin-fixed, paraffin-embedded utero/placental and CL samples 2-3 µm thick were cut with a microtome and mounted on SuperFrost Plus microscope slides (Menzel-Gläser, Braunschweig, Germany). The subsequent experimental procedure was according to our

previously described protocol (Kowalewski et al., 2006a; Kowalewski et al., 2006b). Briefly: slides were deparaffinized in xylol, rehydrated in a graded ethanol series and washed under running tap water for 5 min. Antigen retrieval was performed in 10mM citrate buffer pH 6.0 at room temperature for 5 min followed by microwave irradiation in an oven run at 560 W for 15 min. Then, sections were cooled at room temperature for 20 min and washed under the running tap water for 5 min. Endogenous peroxidase activity was quenched by incubating the sections in 0.3% hydrogen peroxide in methanol for 30 min on a shaker. Afterwards, they were washed in IHC buffer/0.3% Triton X, pH 7.2-7.4 (0.8mM Na_2HPO_4, 1.47mM KH_2PO_4, 2.68 mM KCl, 137 mM NaCl for 5 min and incubated in 10% goat serum for 20 min at room temperature in order to block non-specific binding. Thereafter, slides were overlaid with canine-specific, polyclonal, affinity purified guinea pig anti-PGFS and anti-PGDH antibodies, the same as for western blot analysis. Both antibodies were diluted 1:750 in IHC/0.3% Triton X buffer and incubated overnight at 4°C. The following negative controls were used: slides omitting the primary antibody and slides incubated with pre-immune guinea pig serum at the same dilution as the primary antibodies. After subsequent washing with IHC/0.3% Triton X buffer, tissue sections were incubated for 30 min at room temperature with biotinylated secondary, goat anti-guinea pig antibodies at 1:100 dilution (Vector Laboratories, Burlingame, USA). Signals were enhanced with the streptavidin-avidin-peroxidase Vectastain ABC kit (Vector Laboratories), for 30 min at room temperature and finally detected using the Liquid DAB+ substrate kit (Dako Schweiz AG, Baar, CH). Sections were counterstained with haematoxylin, washed under running tap water, dehydrated in a graded ethanol series and embedded in Histokit (Assistant, Osterode, Germany).

6.9 In situ Hybridization (ISH)

RT-PCR reactions with the following primers were performed to generate templates for subsequent cRNA probe synthesis: *15PGDH* forward: 5'-GCC CTG GAT GAG CAG TTT G-3', *15PGDH* reverse: 5'-CCA CCT TCT CCT CCG TTT TG-3'. Length of the amplicons was 251bp. The PCR products were separated on a 2% ethidium bromide-stained agarose gel, purified using the Qiaex II gel extraction system (Qiagen GmbH Hilden, Germany) and subcloned into the pGEM-T vector (Promega). Selected clones were control-digested with NcoI and NotI restriction enzymes (New England Biolabs) and sent for sequencing (Microsynth). After linearizing plasmids with the respective restriction enzymes: NcoI for antisense cRNA (anti-sense probe) and NotI for the sense cRNA (sense probe), synthesis of DIG-labelled probes was performed with the DIG-RNA labelling kit from Roche Diagnostics. Dot-blot analysis of serial dilutions of DIG-labelled cRNA was performed on positively-charged Nylon Membrane (Roche Diagnostics). For nonradioactive ISH, paraffin-embedded tissue sections were used. Utero/placental samples were cut with a microtome (sections 2-3 μm thick) and mounted on SuperFrost Plus microscope slides (Menzel-Gläser), dewaxed, rehydrated, digested with proteinase K (70μg/ml; Sigma-Aldrich) for permeabilization, post-fixed with 4% paraformaldehyde and submitted to the ISH procedure following our previously described protocol (Kowalewski et al., 2006a). The hybridization was overnight at 37°C. The DIG-labelled cRNA was detected via alkaline phosphatase conjugated sheep anti-DIG Fab Fragments (Roche Diagnostics) used at 1:5,000 dilution in 1% ovine serum according to the manufacturer's instructions. Signals were detected with the substrate 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (NBT/BCIP; Roche Diagnostics).

6.10 Statistics

Parametric one-way analysis of variance (ANOVA) followed by a Tukey-Kramer multiple comparisons post-test were used to determine the effect of time on luteal *15PGDH* mRNA expression throughout pregnancy. Due to the uneven distribution of the data obtained for the utero/placental *15PGDH* mRNA expression and the luteal 15PGDH protein expression, the Kruskal-Wallis test (a nonparametric ANOVA) followed by Dunn's multiple comparison test were performed in the event of $P<0.05$. The numerical data are presented as the mean ± standard deviation (S.D.). All tests were performed with the statistical software program GraphPad 3.06 (GraphPad Software, San Diego, CA, USA).

7 Results

7.1 Utero/placental and luteal expression of PGFS throughout pregnancy

Canine PGFS-His and EYFP-tagged fusion proteins were overexpressed in Vero 2.2 cells. The expression of PGFS-EYFP protein was controlled under a fluorescence microscope to check for transfection efficiency (Fig. 1A,B). When high transfection efficiency was observed (at 24-48h), cells were harvested and used in western blot analysis to detect recombinant proteins using the anti-EYFP and anti-His antibodies (Fig. 1C). The PGFS-EYFP protein was detected as a protein of approximately 64kDa, whereas the PGFS-His displayed a molecular weight of approximately 34-36kDa, thereby indicating the actual size of the canine PGFS protein cloned using the canine-specific cDNA sequence with GenBank number AY875970 (Fig. 1C) as a template. In a further step, western blot analysis with the custom-made canine specific anti-PGFS antibody was performed. The PGFS-His coupled protein was used as a positive control (Fig. 1D). The apparent molecular size of the expressed protein was identical to that of the endogenous isoform expressed in the canine utero/placental compartments (Fig. 1D). Non-transfected Vero 2.2 cells were used as negative controls; only a weak background signal was observed (Fig. 1D). Blocking the PGFS antibody with epitope-specific blocking peptide significantly diminished the signal (Fig. 1E).

The utero/placental expression of PGFS protein throughout pregnancy was strongly modulated and dependent upon the stages of pregnancy. There were no or only weak signals observed on western blots prior to implantation (Fig. 1F). This was probably attributed to the very low expression level observed at this time. Afterwards, during the post-implantation and mid-gestation periods of pregnancy, PGFS protein expression was strongly upregulated (Fig. 1F). The signal intensity decreased at the time of the prepartum luteolysis (Fig. 1F).

Only weak or no PGFS protein signals were observed in samples derived from luteal tissues (Fig. 1G).

The utero/placental expression of PGFS was localized by IHC using the canine-specific anti-PGFS antibody. Within the utero/placental units, PGFS was localized in the epithelial cells of the superficial uterine glands, the so-called 'glandular chambers' (Fig. 2A). Interestingly, no signals were observed in the deep uterine glands (Fig. 2 B). The placental signals were localized in the trophoblast cells (Fig. 2 C,D), mostly within the feto-maternal contact zone (Fig. 2 D). No increase was observed at the time of parturition.

7.2 Spatio-temporal expression of 15PGDH in utero/placental compartments and CL throughout pregnancy

Following homology cloning, the predicted ORF of canine *15PGDH* of 801 nucleotides encoding for a 267 amino acid protein was sequenced and characterized. As revealed in the homology search against the GenBank database with the online available BLAST software, the canine *15PGDH* sequence displays a very high similarity for nucleotide (91-95%) and protein sequences (94-97%) with those of, e.g., horse (GenBank accession number: NM_001081786), pig (GenBank accession number: NM_001190248), cow (GenBank accession number: NM_001034419) or human (GenBank accession number: NM_000860). In the alignment against the canine genomic sequence, the *15PGDH* has been localized to chromosome 25. The cloning and characterization of canine *15PGDH* provided the basis for our subsequent investigations at the mRNA and protein levels.

The expression of *15PGDH* mRNA was detected in all luteal and utero/placental samples investigated and demonstrated highly significant effects of time ($P < 0.0003$ and $P < 0.0027$ in the CL and utero/placental units, respectively) (Fig. 3A,B). As for the utero/placental compartment, the lowest expression was observed pre-implantation. Following placental

formation, levels of *15PGDH* mRNA increased significantly ($P<0.05$), reaching the highest mRNA levels during the post-implantation period of pregnancy. Afterwards, the mRNA levels started to fall gradually reaching their lowest levels during the prepartum luteolysis ($P< 0.05$) (Fig. 3A).

Similarly, the luteal *15PGDH* mRNA levels increased from the pre-implantation stage of gestation to reach significantly higher values ($P<0.05$) post-implantation. However, no significant changes were observed thereafter from mid-gestation through prepartum luteolysis ($P>0.05$) (Fig. 3B).

Applying the same experimental strategy, based on the cloned canine-specific *15PGDH* sequence (GenBank: JQ407019), 15PGDH-His and -EYFP-tagged fusion proteins were produced in a heterologous system. The expression of 15PGDH-EYFP was visualized under the fluorescence microscope (Fig. 4 A,B). Subsequently, after 24-48h, cells were harvested and used in western blot analysis for detection of fusion proteins (Fig. 4 C). The 15PGDH-EYFP was found as a protein of approximately 57kDa; the molecular weight of 15PGDH-His was approximately 30kDa, indicating the size of the cloned 15PGDH (Fig. 4 C). When the canine specific anti-15PGDH antibody was used, the molecular size of the expressed recombinant protein was identical to that of the endogenous isoform found in the luteal and utero/placental tissues (Fig. 4 D). The 15PGDH-His protein served as a positive control in these experiments. For the negative control, non-transfected Vero 2.2 cells were used, displaying a weak background staining (Fig. 4 D). The signal was significantly diminished when the anti-15PGDH antibody was blocked with epitope-specific blocking peptide (Fig. 4E).

Subsequently, the 15PGDH protein expression profiles were investigated by western blot analysis (Fig. 5). The utero/placental expression was time-dependent ($P=0.0051$). It was highest from the pre-implantation until mid-gestation stages, then there was a significant decrease at prepartum luteolysis ($P<0.05$). At the cellular level, and as determined by IHC, in

the pre-implantation group weaker uterine signals were observed in the surface epithelium and superficial endometrial glands (Fig. 6 A,B). In contrast, strong immunohistochemical signals were observed in the deep uterine glands (Fig. 6B). Following implantation, endometrial signals were localized to the superficial glandular epithelial cells, within the so-called glandular chambers (Fig. 6 C). Placental signals were localized in the trophoblast cells, especially those in the feto-maternal contact zone (Fig. 6 D). A similar signal distribution pattern was found at the mRNA-level by in situ hybridization (Fig. 7).

As for the luteal tissues, western blot signals for 15PGDH expression were generally weaker (Fig. 5 B). Even though the signals tended to be stronger at the earlier luteal stages, when statistical analysis was applied, due mostly to the large individual variations, the luteal protein expression did not differ significantly over time (P=0.3911). The cellular signals were observed throughout pregnancy and were localized to the luteal cells (Fig. 6 E). However, as observed in the western blot analysis, variability between individual animals was high.

7.3 Biochemical activities of canine PGFS and 15PGDH

The recombinant PGFS-His and 15PGDH-His proteins were used in order to determine biochemical activities of the canine proteins towards their specific substrates. The only metabolic activity of PGFS was towards the conversion of PGH2 to PGF2alpha; no activity towards PGE2 synthesis was found. The catabolic activity of the recombinant 15PGDH was confirmed towards both PGF2alpha and PGE2. The PGFS and 15PGDH activities were lacking in the absence of the respective cofactors (NADPH and NAD^+). For both enzymes, samples omitting recombinant proteins were run to check for possible background signals resulting from the assay. These were then subtracted from the signals obtained from enzymatic activity of the recombinant proteins (Fig. 8).

8 Discussion

In spite of the distinct role of PGF2alpha in the acute luteolysis prior to parturition, mechanisms regulating its generation in pregnant dogs are not well understood. Recently, expression of the key-factors in the supply of prostaglandins has been investigated in canine reproductive organs. From the strongly upregulated PTGS2 expression in the placenta fetalis during luteolysis and its cellular colocalization with the mRNA encoding for *PGFS*, *PGES* and the respective prostaglandin receptors, fetal trophoblast cells were suggested as the major source of prostaglandins at term in the dog (Kowalewski et al., 2010). This hypothesis fits well with the observations by Luz et al., 2006 (Luz et al., 2006), who postulated the canine placenta as the main origin of the luteolytic PGF2alpha. Surprisingly, especially in the context of the prepartum luteolysis, the most striking observation from these earlier studies was the decreased *PGFS* mRNA expression at the prepartum luteolysis. However, the respective data at the protein level were missing, thus preventing any further conclusions. In the present study, using the recombinantly expressed proteins, the enzymatic activity of the previously cloned canine *PGFS* towards PGF2alpha synthesis was confirmed. Especially due to the very high sequence homology between *PGFS* and several members of the aldo-keto reductases family, these experiments provided final confirmation of the protein's biochemical identity. Furthermore, the canine-specific antibody was synthesized, validated and applied to investigate the expression and cellular localization of PGFS in the canine CL and utero/placental compartments. The uterine expression of PGFS was localized to the surface epithelial cells and the superficial glandular chambers after implantation. The low uterine expression of PGFS prior to implantation further supports the previously published data (Kowalewski et al., 2010) but this time at the molecular level, precluding uterine PGF2alpha as the possible endocrine factor influencing luteal function in the bitch. Similarly, an auto/paracrine function of locally produced PGF2alpha acting as a luteolytic factor is most

unlikely, since the luteal expression of the PGFS protein was very low throughout gestation. This is in agreement with our previous hypothesis that the slow luteal regression in the dog is independent of the locally produced PGF2alpha (Kowalewski et al., 2008) and also reflects the low PGF2alpha output observed *in vitro* in luteal tissue explants collected at the late CL-stage (Luz et al., 2006).

Interestingly, at the cellular level strong utero/placental PGFS signals first became visible after implantation and coincided with its increased placental expression. Staining was localized to the fetal trophoblast cells, especially in the feto-maternal contact zone, strongly implying the possible role of PGFS during the processes of decidualization, placental development and, taking into consideration the placental PGFS expression, likely also during trophoblast invasion.

Yet, with regards to the prepartal luteolysis, the most striking observation from the present study was the decreased utero/placental expression of PGFS protein at the time of prepartum luteolysis. On the one hand, it apparently confirms our previous data on the *PGFS* gene transcriptional activity, on the other hand, however, let us to further speculate on the molecular endocrine mechanisms involved in regulation of the provision of the luteolytic PGF2alpha in the bitch. Thus, possible alternative biosynthetic pathways for the enzymatic PGFS-activity, or increased substrate (i.e., PGH2) availability as a result of the upregulated PTGS2 expression, still need to be considered.

After observing the deviating patterns of PGFS expression and the PGFM profile in peripheral blood (Nohr et al., 1993; Kowalewski et al., 2010), we felt prompted to investigate the expression of 15PGDH, which is another factor potentially capable of functional modulation of PGF2alpha activity at the post-transcriptional level. The canine-specific *15PGDH* sequence was not characterized prior to our study. Thus, molecular cloning and sequencing were prerequisites to obtaining the subsequent data on its expression and cellular localization. As for PGFS, the biochemical activity of the recombinantly expressed canine

15PGDH towards its specific substrates (PGE2 and PGF2alpha) was confirmed. Detailed studies on enzyme kinetics were not within the focus of the present study.

Canine-specific anti-15PGDH antibody was generated, validated and used in the experiments reported here. Interestingly, especially at the protein level and as revealed in western blot analysis, the utero/placental 15PGDH-expression was upregulated until mid-gestation, followed by a prepartum decrease. The cellular localization reflected that of the PGFS protein. This correlation strongly implies a functional interrelationship between these two factors and could explain the low PGF2alpha levels observed in peripheral blood at the time of increased PGFS expression (Kowalewski et al., 2010). Recently, a similar regulatory mechanism has been described for the murine model (Winchester et al., 2002) in which the prepartal PGF2alpha increase was associated with decreased uterine 15PGDH expression.

Another intriguing observation from the present study was the apparent compartmentalisation of the uterine *15PGDH* mRNA and protein expression prior to implantation with stronger signals observed in the deep uterine glands. A possible meaning of this phenomenon could be in preventing the passage of locally-produced PGF2alpha from endometrium to myometrium, additionally contributing towards the gestational myometrial quiescence. Such a regulatory mechanism has been recently proposed for mice; the decline of uterine 15PGDH during late gestation appears to be responsible for the facilitated PGF2alpha transfer towards the myometrium where it can be involved in the initiation of muscular contractility (Winchester et al., 2002).

In contrast to the strongly time-dependent utero/placental 15PGDH expression, the luteal protein expression varied strongly among the samples and groups. Even if the protein levels tended to be higher at the beginning of the CL-phase, they did not change significantly throughout gestation. Especially since the luteal *15PGDH* mRNA levels displayed a time-

related expression pattern, further investigations, possibly involving larger experimental groups, are needed in order to draw any final conclusion.

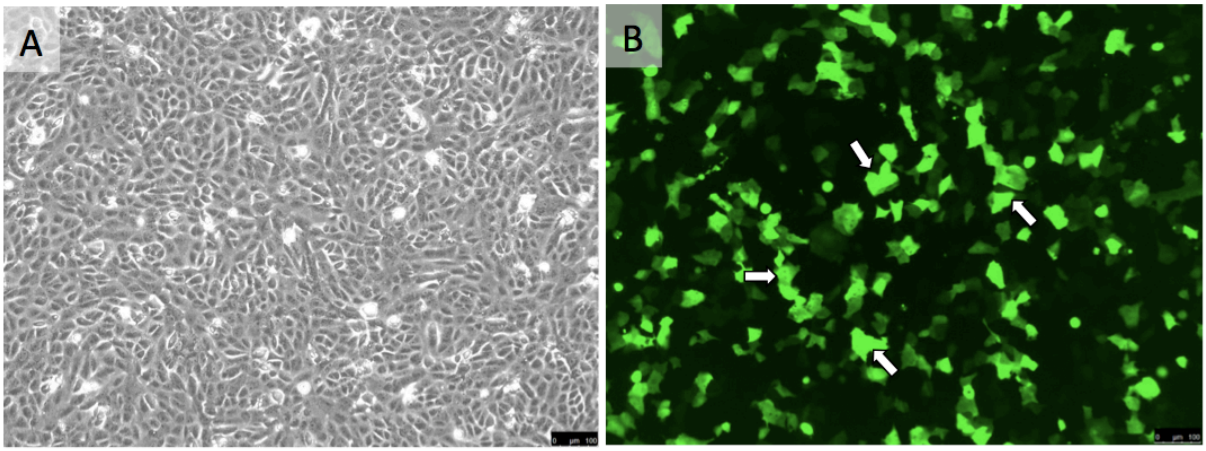
Previously reported observations made in humans indicate that placental *15PGDH* mRNA expression and activity is elevated by the availability of P4 analogues such as R5020 and medroxyprogesterone acetate (Patel et al., 1999). In addition, treatment with selective P4-receptor blockers, RU486 and onapristone, significantly reduced 15PGDH activities in human syncytiotrophoblast and chorionic trophoblast in vitro (Patel et al., 1999). Taking this into consideration, together with the fact that placental 15PGDH expression in the dog strongly follows the prepartum P4 decrease, it appears plausible that also in the canine species the expression of 15PGDH could be P4-dependent. Observations from our parallel study corroborate this hypothesis: the utero/placental *15PGDH* expression in mid-pregnant dogs was significantly reduced in response to treatment with the antigestagen aglepristone (own data, not published).

Taken together, by describing the expression of PGFS and 15PGDH the present study provides a basis for better understanding the physiological mechanisms regulating the supply of prostaglandins during canine pregnancy and, importantly, especially at the acute prepartum luteolysis. The biochemical activity of the previously sequenced canine *PGFS*, as well as of the freshly cloned *15PGDH*, towards their specific substrates was confirmed. Canine-specific custom made antibodies were generated and tested, providing a useful tool for further studies. Amongst the most interesting findings with possible functional implications are: 1) the strongly upregulated utero/placental expression of PGFS during the post-implantation and mid-gestation stages of pregnancy, which implies its involvement in placental development and maintenance, and 2) the coincident expression and localization of 15PGDH together with PGFS suggesting a modulatory role of 15PGDH as a “gate-keeper” controlling locally the supply of prostaglandins from the utero/placental compartment in the dog.

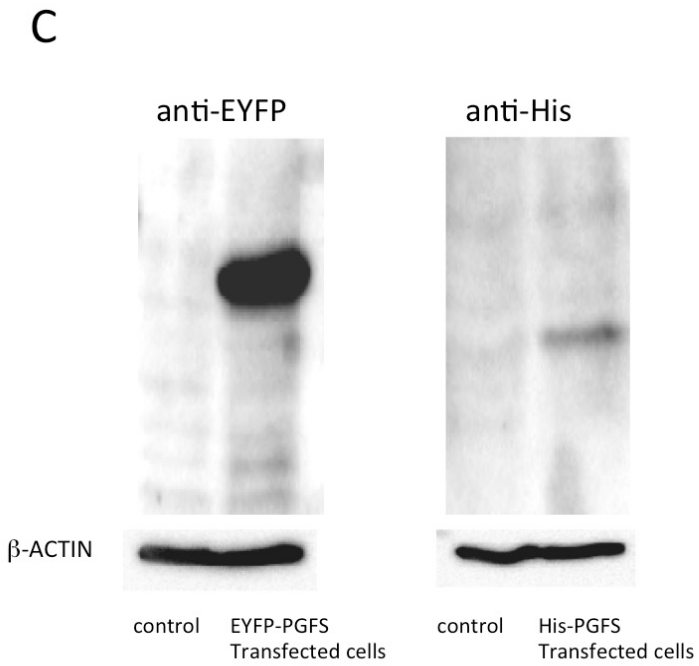
9 Figures and Legends

9.1 Figure 1

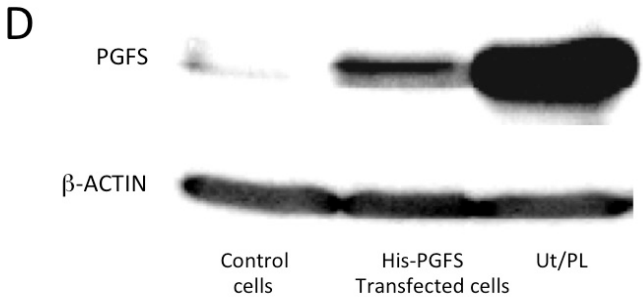
Transfection of Vero cells with pHSV-EYFP-Rfc-C1-PGFS (for EYFP-PGFS recombinant fusion-protein expression) and pHSV-V5His-Rfc-C1-PGFS (for HIS-PGFS recombinant fusion-protein expression) vectors; validation of canine-specific anti-PGFS antibody and PGFS protein expression profiles in the utero/placental (Ut/PL) compartments and corpus luteum (CL) throughout pregnancy.



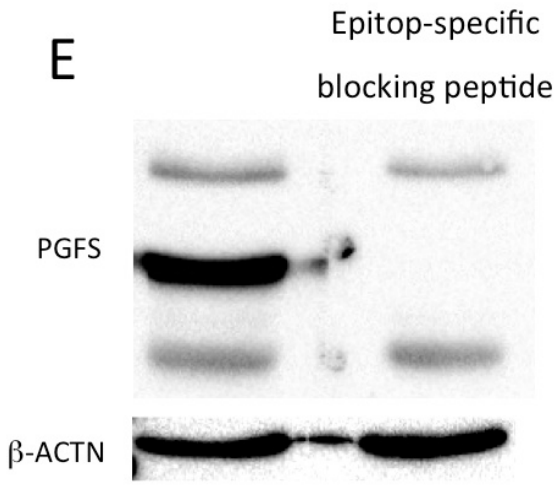
(A) Appearance of transfected Vero 2.2 cells under 20x objective in bright light microscopy. (B) EYFP-PGFS transfected Vero 2.2 cells; the white arrows indicate the fluorescing signals of cells expressing the recombinant fusion- protein.



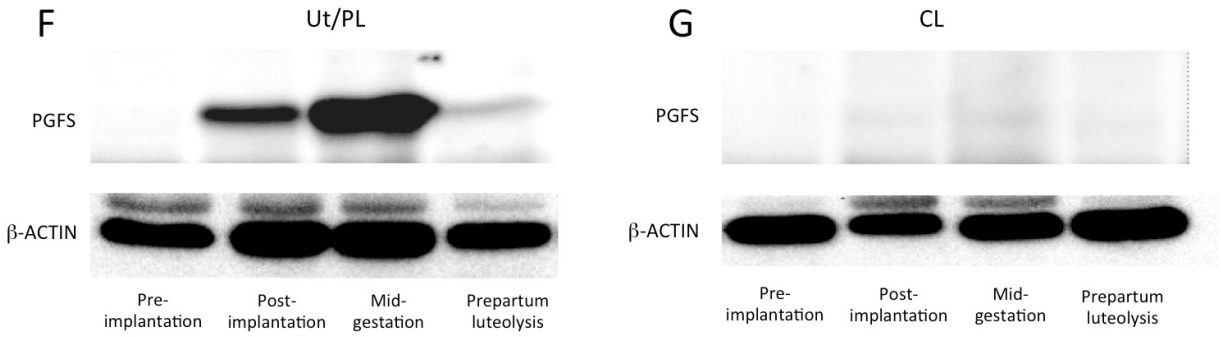
(C) The expression of EYFP-PGFS (approx. 64 kDa) and His-PGFS (approx. 34-36 kDa) fusion proteins in Vero 2.2 cell lysates was examined by western blot analysis using the anti-EYFP and anti-His antibodies. As a negative control, non-transfected Vero 2.2 cells lysate was used; β-actin (45 kDa) was used as an internal loading control.



(D) Western blot analysis of His-PGFS recombinant protein expression in Vero 2.2 cells lysate compared with the utero/placental sample from the mid-gestation period of pregnancy (approx. 34-36 kDa); proteins were stained with the canine-specific anti-PGFS antibody. Non-transfected Vero 2.2 cells were used as a negative control. β-ACTIN (43 kDa) served as an internal loading control.



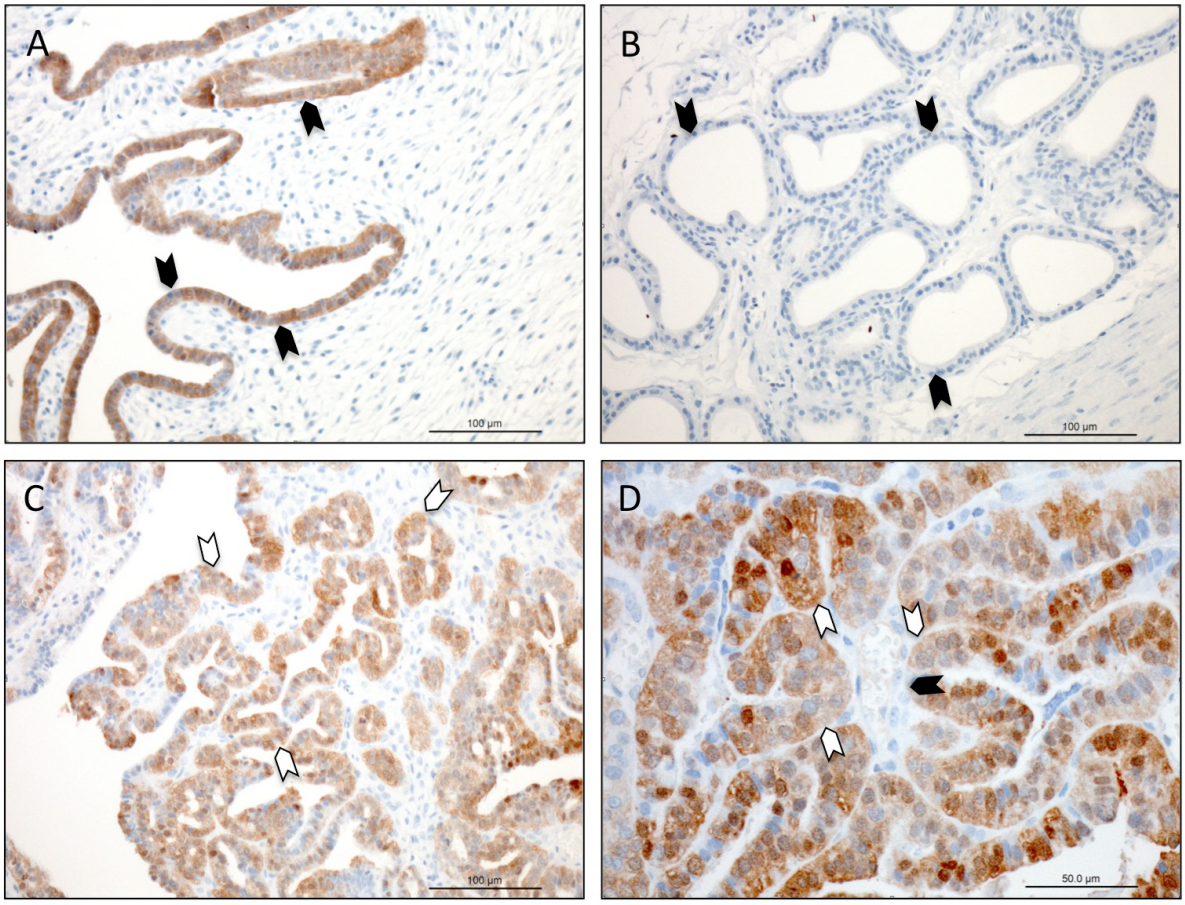
(E) The epitope-specific blocking peptide was used in order to quench the PGFS-specific signal.



(F) and (G) 20µg tissue homogenates were used in western blot analysis of PGFS expression in utero/placental compartments and CL throughout pregnancy. Representative immunoblots are shown.

9.2 Figure 2

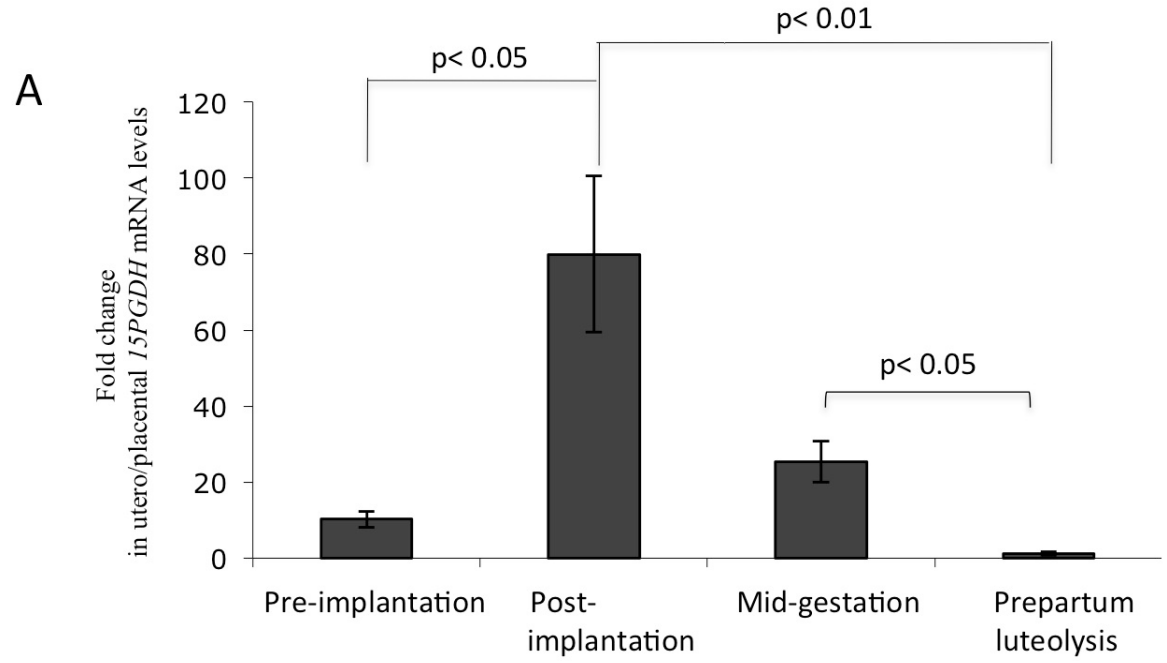
Immunohistochemical (IHC) localization of PGFS in the utero/placental compartment during post-implantation (A-C) and at mid-gestation (D).



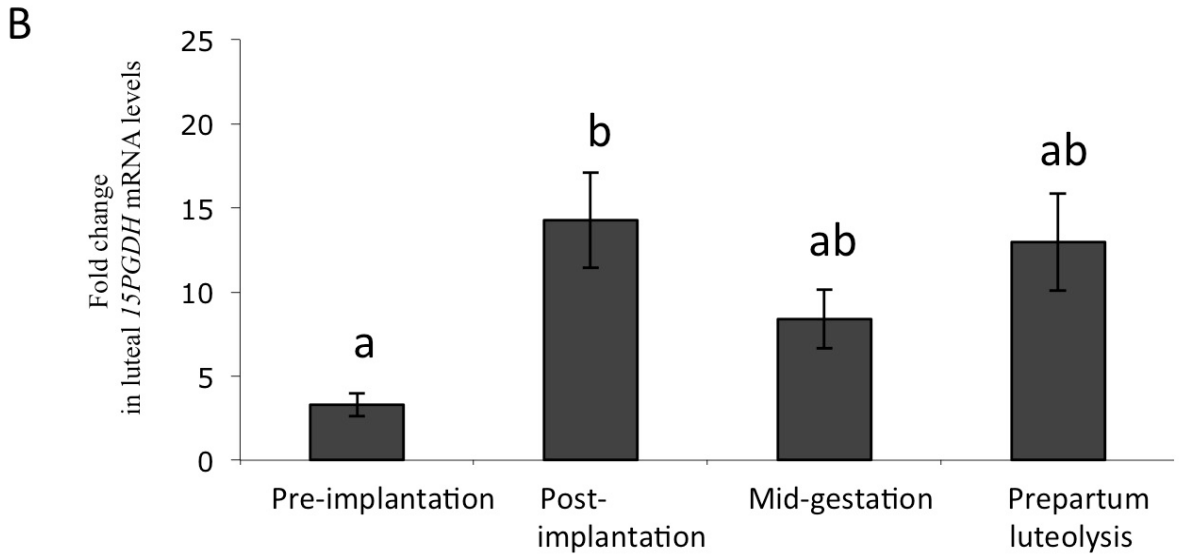
(A) IHC signals are localized to the epithelial cells of the superficial endometrial glands (solid arrowheads) (the so-called glandular chambers). (B) Deep uterine glands (solid arrowheads) stain negatively for PGFS. (C,D) In the placental labyrinth, fetal trophoblast stains strongly for PGFS (open arrowheads). No signals are localized within the placenta materna, including maternal blood vessels (solid arrowhead).

9.3 Figure 3

Time dependent expression of canine *15PGDH* as determined by Real Time (TaqMan) PCR.



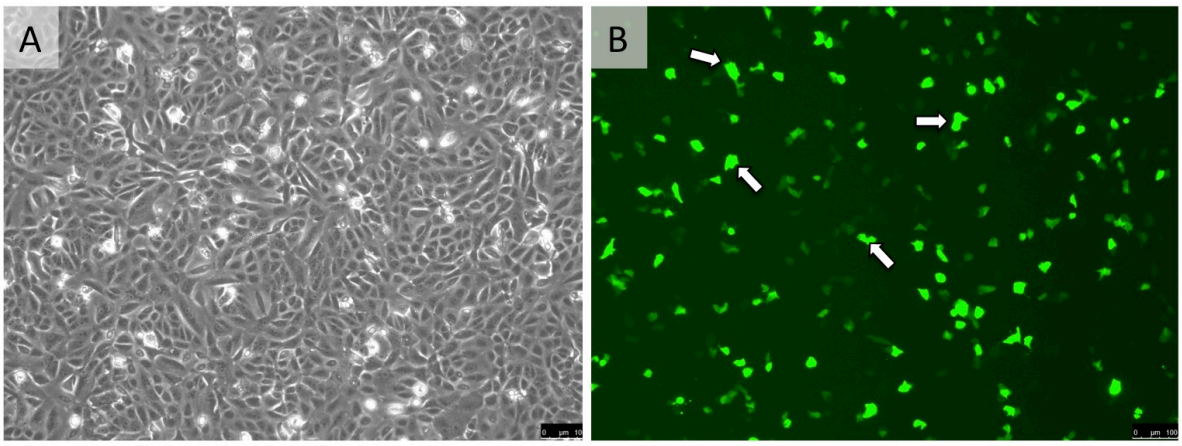
(A) Utero/placental compartments throughout pregnancy (mean ± S.D.).



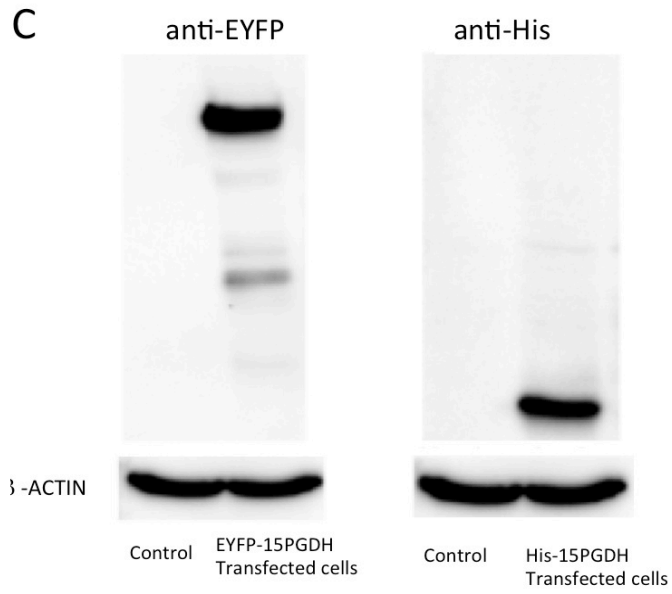
(B) CL of pregnancy (mean ± S.D.). Bars with different letters differ at P<0.05.

9.4 Figure 4

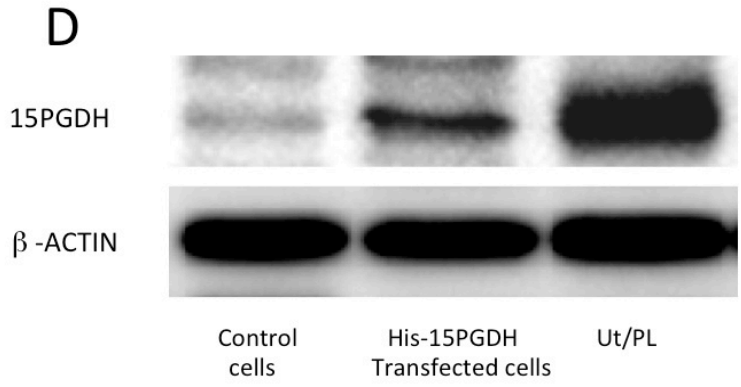
Transfection of Vero cells with pHSV-EYFP-Rfc-C1-15PGDH (for EYFP-15PGDH recombinant fusion-protein expression) and pHSV-V5His-Rfc-C1-15PGDH (for His-15PGDH recombinant fusion-protein expression) vectors and validation of canine-specific anti-15PGDH antibody.

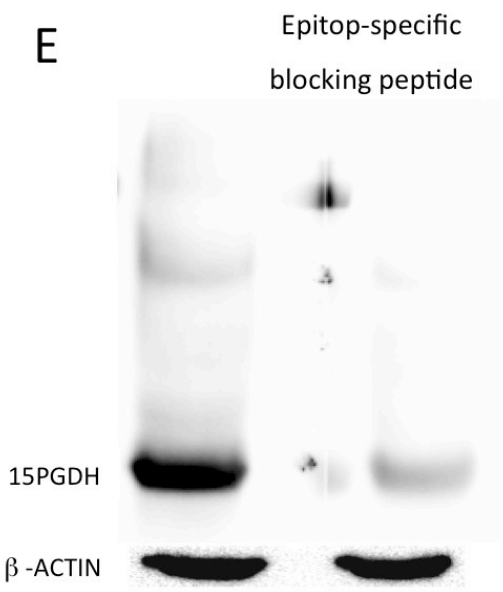


(A) Appearance of transfected Vero 2.2 cells under 20x objective in bright light microscopy. (B) EYFP-15PGDH transfected Vero 2.2 cells; the white arrows indicate the fluorescing signals of cells expressing the recombinant fusion protein.



(C) The expression of EYFP-15PGDH (approx. 57 kDa) and His-15PGDH (approx. 30 kDa) fusion proteins in Vero 2.2 cell lysates was examined by western blot analysis using the anti-EYFP and anti-His antibodies. As a negative control, non-transfected Vero 2.2 cells lysate was used; β-ACTIN (45 kDa) was used as an internal loading control.

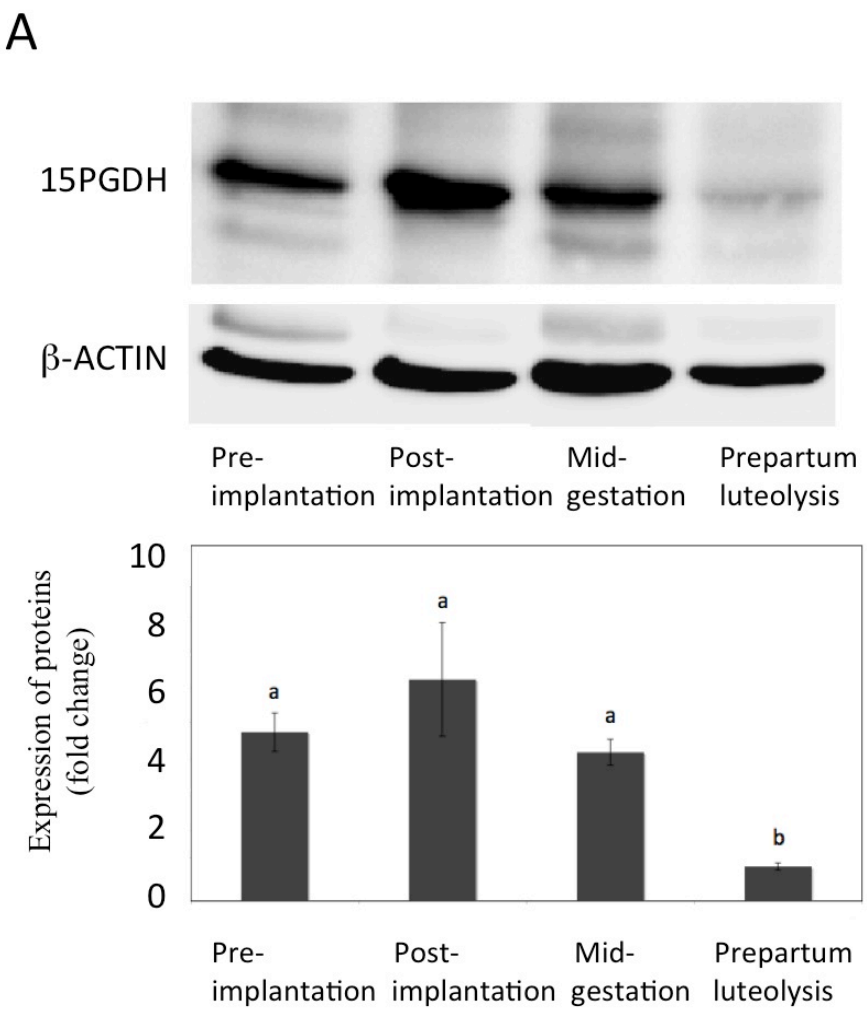


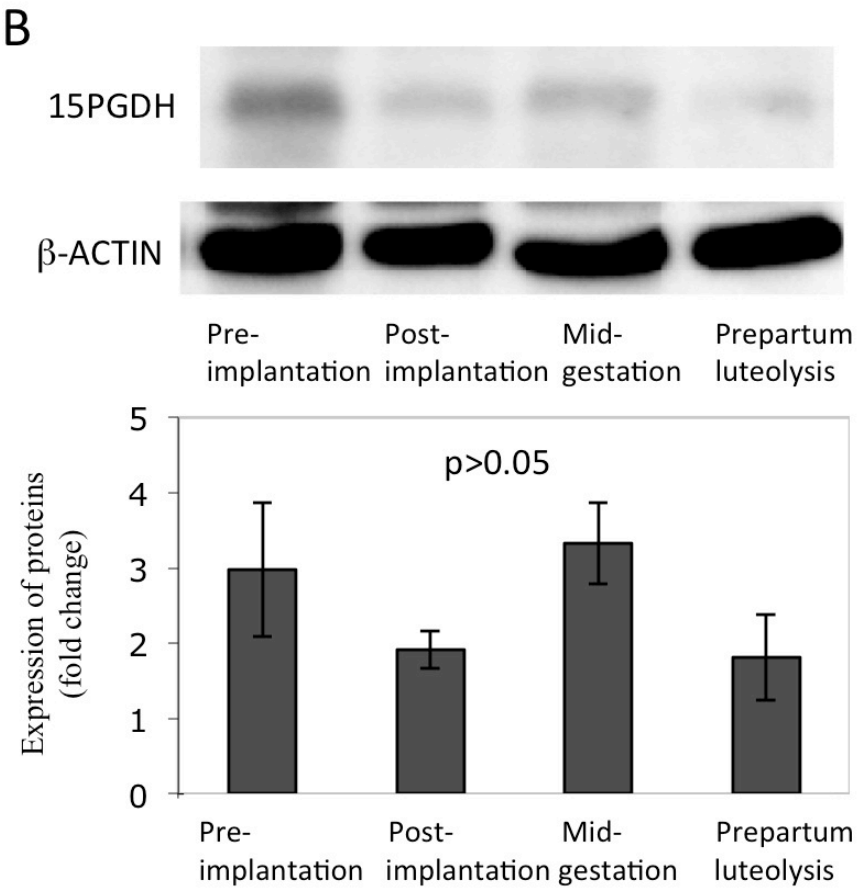


(D) Western blot analysis of His-15PGDH recombinant protein expression in Vero 2.2 cell lysates compared with utero/placental samples from the mid-gestation period of pregnancy (approx. 30 kDa); proteins were stained with the canine-specific anti-15PGDH antibody. Non-transfected Vero 2.2 cells used as negative controls revealed a low background signal. β-ACTIN (45 kDa) served as an internal loading control. (E) The epitope-specific blocking peptide was used to quench the 15PGDH-specific signal.

9.5 Figure 5

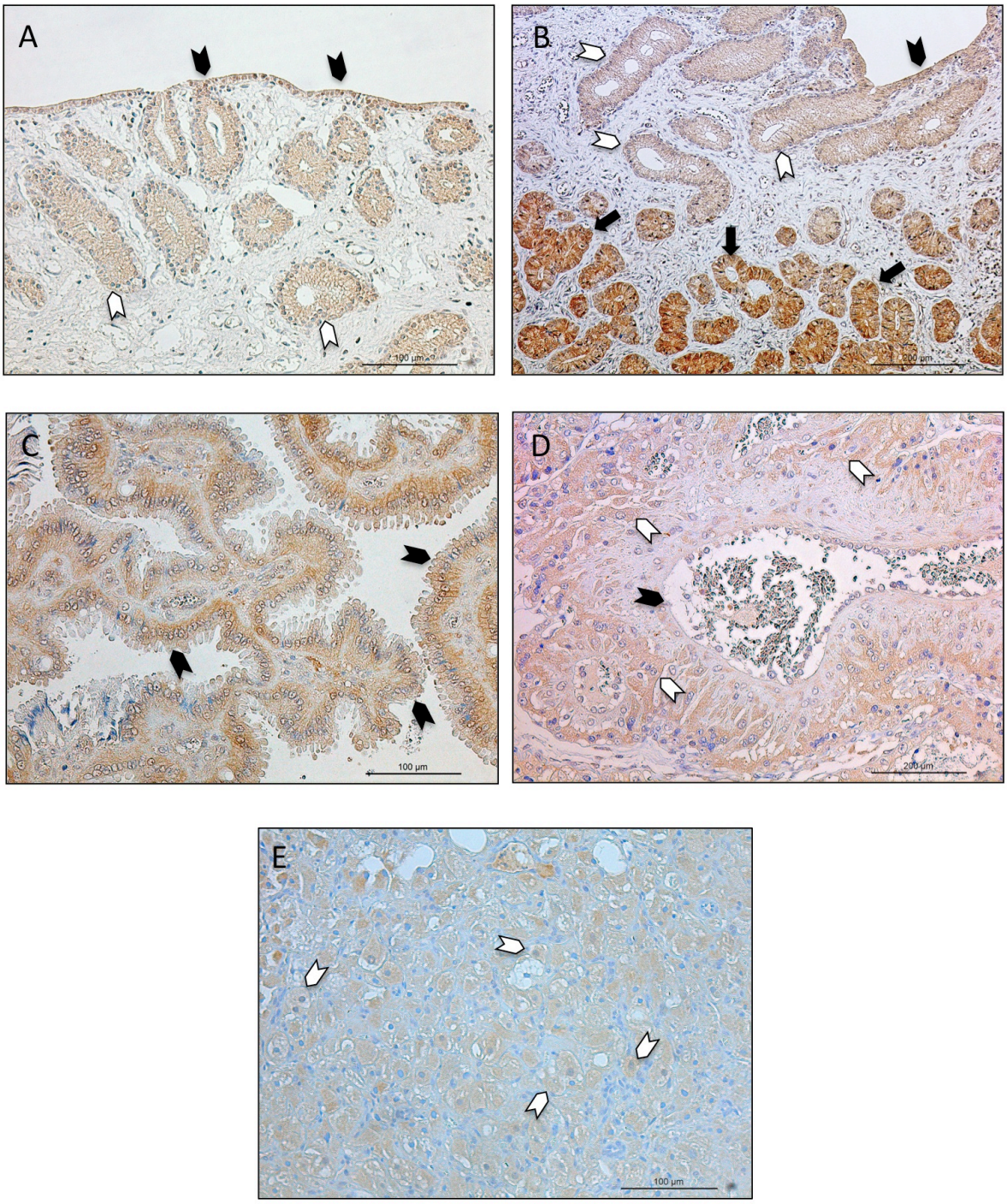
Time-dependent expression of 15PGDH protein in the canine utero/placental compartment (A) and CL (B) throughout pregnancy. 20µg tissue homogenates were used in western blot analysis. Representative immunoblots are shown. Lower panels represent densitometric values (integrated optical density) for 15PGDH normalized against β-ACTIN (mean ± S.D). Bars with different letters in (A) differ at $P<0.05$.




B
15PGDH
β-ACTIN
Pre-implantation
Post-implantation
Mid-gestation
Prepartum luteolysis
p>0.05
Expression of proteins (fold change)
0
1
2
3
4
5
Pre-implantation
Post-implantation
Mid-gestation
Prepartum luteolysis

9.6 Figure 6

Immunohistochemical (IHC) localization of 15PGDH in the uterus pre-implantation (A,B), in the utero/placental compartment at mid-gestation (C,D) and in luteal samples (E).

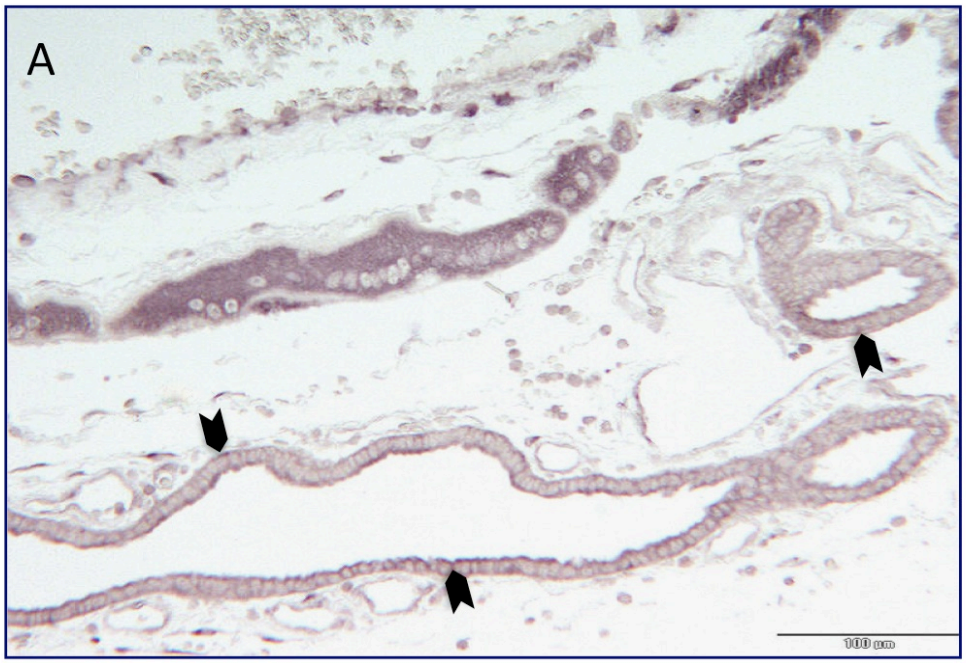


(A,B) Pre-implantation endometrial 15PGDH expression is localized to the surface epithelial cells (solid arrowheads) and to the glandular epithelial cells of the superficial (weaker signals; open arrowheads) and deep (stronger signals; solid arrows) uterine glands. (C,D) Within the

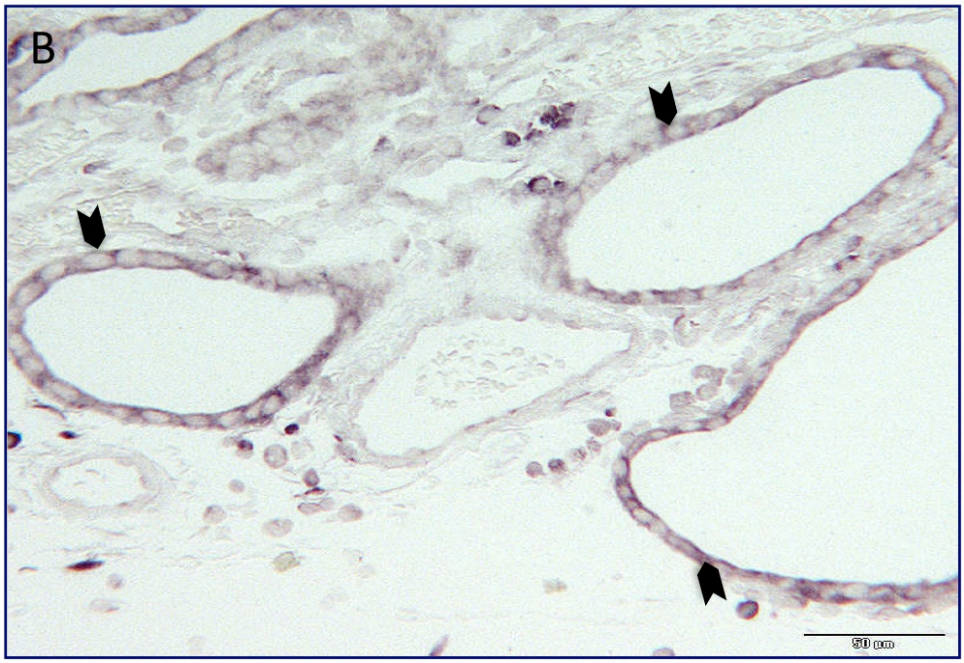
utero/placental compartment, signals are localized also to the superficial uterine glands (the so-called glandular chambers) (solid arrowheads in C) and to the fetal trophoblast cells (open arrowheads in D) at the feto-maternal contact zone, mostly in the strongly invading trophoblast cells surrounding maternal blood vessels (solid arrowheads in D). The luteal expression of 15PGDH is targeted solely to the luteal cells (E; canine CL of mid-pregnancy shown).

9.7 Figure 7

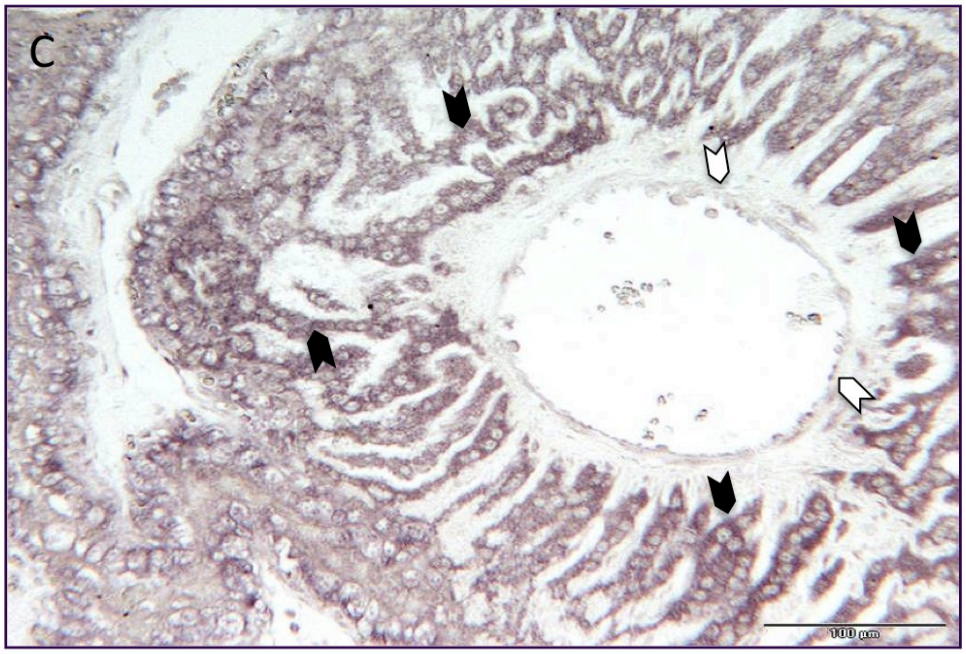
Localization of *15PGDH* mRNA in the utero/placental compartment of a mid-pregnant bitch by *in situ* hybridization (ISH).



(A) Signals are localized to the superficial (solid arrowheads) uterine glands.



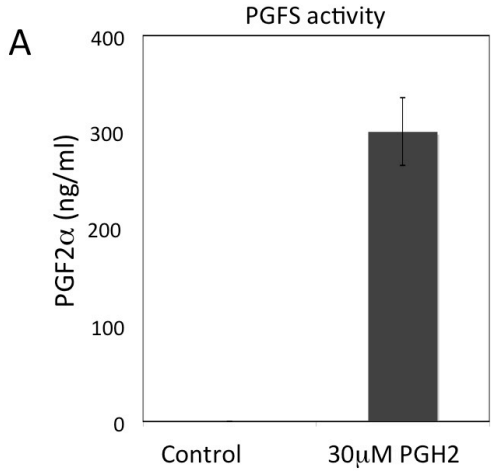
(B) Signals are localized to the deep (solid arrowheads) uterine glands.



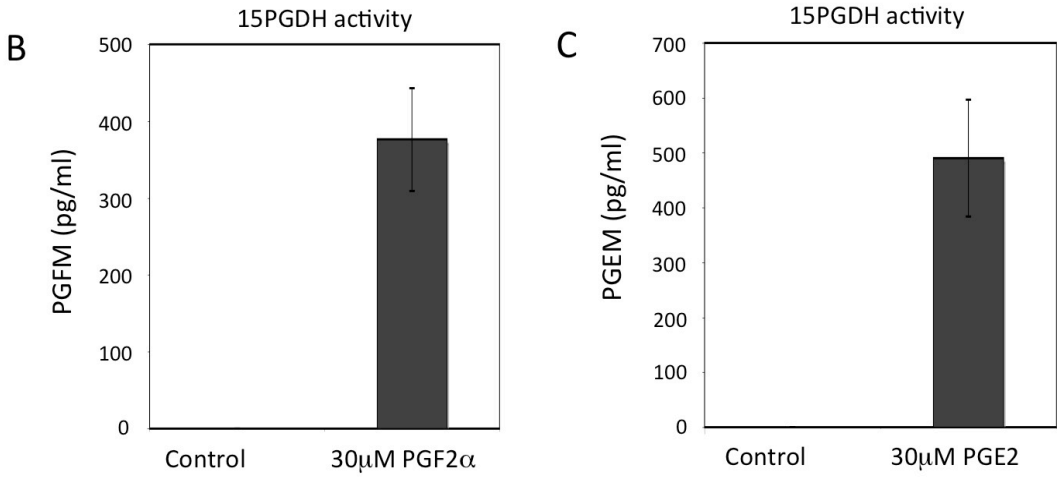
(C) Placental signals are localized in the fetal trophoblast cells (solid arrowheads), especially in the strongly invading trophoblast cells surrounding maternal blood vessels (open arrowheads).

9.8 Figure 8

Recombinant canine PGFS and 15PGDH activities as described in Materials and Methods.



(A) Depicted is the conversion of PGH2 (30μM) to PGF2alpha due to PGFS activity in the presence (1μg) or absence (control) of recombinant PGFS.



(B,C) The conversion is shown of PGF2alpha (30μM) and PGE2 (30μM) to PGFM and PGEM, respectively, due to 15PGDH activity in the presence (1μg) or absence (control) of recombinant protein.

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